

## Interactions among CotB, CotG, and CotH during Assembly of the *Bacillus subtilis* Spore Coat

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Spores formed by wild-type *Bacillus subtilis* are encased in a multilayered protein structure (called the coat) formed by the ordered assembly of over 30 polypeptides. One polypeptide (CotB) is a surface-exposed coat component that has been used as a vehicle for the display of heterologous antigens at the spore surface. The *cotB* gene was initially identified by reverse genetics as encoding an abundant coat component. *cotB* is predicted to code for a 43-kDa polypeptide, but the form that prevails in the spore coat has a molecular mass of about 66 kDa (herein designated CotB-66). Here we show that in good agreement with its predicted size, expression of *cotB* in *Escherichia coli* results in the accumulation of a 46-kDa protein (CotB-46). Expression of *cotB* in sporulating cells of *B. subtilis* also results in a 46-kDa polypeptide which appears to be rapidly converted into CotB-66. These results suggest that soon after synthesis, CotB undergoes a posttranslational modification. Assembly of CotB-66 has been shown to depend on expression of both the *cotH* and *cotG* loci. We found that CotB-46 is the predominant form found in extracts prepared from sporulating cells or in spore coat preparations of *cotH* or *cotG* mutants. Therefore, both *cotH* and *cotG* are required for the efficient conversion of CotB-46 into CotB-66 but are dispensable for the association of CotB-46 with the spore coat. We also show that CotG does not accumulate in sporulating cells of a *cotH* mutant, suggesting that CotH (or a CotH-controlled factor) stabilizes the otherwise unstable CotG. Thus, the need for CotH for formation of CotB-66 results in part from its role in the stabilization of CotG. We also found that CotB-46 is present in complexes with CotG at the time when formation of CotB-66 is detected. Moreover, using a yeast two-hybrid system, we found evidence that CotB directly interacts with CotG and that both CotB and CotG self-interact. We suggest that an interaction between CotG and CotB is required for the formation of CotB-66, which may represent a multimeric form of CotB.

During the process of sporulation in the gram-positive soil bacterium *Bacillus subtilis* the developing spore is encased in a complex protein structure called the coat, which confers resistance to several physicochemical agents and contributes to the response of spores to the presence of germinants (7, 8, 15). The coat is formed by over 30 polypeptides, ranging in size from about 6 to about 70 kDa, which are assembled into a lamellar inner coat and a thick electron-dense outer coat (7, 8, 15). With only one possible exception (38), synthesis of the coat structural components is restricted to the mother cell chamber of the sporulating cell and is temporally governed by a cascade of transcription factors in the order  $\sigma^E$ , SpoIIID,  $\sigma^K$ , and GerE (7, 8, 15, 24, 35, 40).  $\sigma^E$  and SpoIIID drive synthesis of a class of morphogenetic proteins that (irrespective of their association with the final coat structure) appear to guide the assembly of several structural components into the spore coat (reviewed in references 7, 8, and 15). For instance, spores produced by a *cotE* mutant fail to assemble the electron-dense outer coat and the remaining coat structure appears to lack, in addition to CotE, several other abundant components (47). The results of a recent study indicate that specific regions in CotE are re-

quired for the assembly of different proteins and suggest that CotE might control the assembly of several outer-coat components by direct protein-protein interactions (2, 28). Most of the coat structural components are synthesized (under the control of  $\sigma^K$  and GerE) at a later stage in coat assembly, and it is only after  $\sigma^K$  is activated that assembly of the coat is unequivocally recognized by electron microscopy of sporulating cells (7, 8, 15). Activation of  $\sigma^K$  results in the expression of several genes coding for spore coat proteins and also results in transcription of the *gerE* gene (4), which encodes an ambivalent transcriptional regulator of coat gene expression. GerE acts together with  $\sigma^K$  to activate a late class of *cot* genes, but it also represses transcription of other *cot* genes (18, 19, 45, 46). These regulatory circuits suggest that the time and level of expression of the genes coding for coat structural components are important for the correct assembly of the coat structure (7, 8, 15). Proper assembly of the coat further relies on mechanisms such as translational control (34) and posttranslational modifications, including proteolytical processing of larger precursors, protein secretion, and protein cross-linking (reviewed in references 7, 8, and 15). These modifications may provide an additional level of control over the timing of assembly of specific components. For example, SafA is a morphogenetic protein of about 45 kDa produced under  $\sigma^E$  control from hour 2 of sporulation onwards but the main form of SafA detected in the coats is a smaller (approximately 30 kDa) species corresponding to the C-termi-

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nal region of the protein (32, 33). This smaller species is produced by internal translation initiation (34). In addition, the full-length and 30-kDa forms of SafA are processed by the YabG protease, which is produced under the control of  $\sigma^K$  (42, 43). The exact contribution of these mechanisms to the ordered assembly of the various coat components is poorly understood, and determination of their nature and contribution will ultimately rely on the functional and structural characterization of selected components (11, 29). To learn more about the mechanisms involved in the morphogenesis of the coat structure, we analyzed the assembly of the outer-coat protein CotB. The *cotB* gene was initially found (by reverse genetics) to encode an abundant spore coat component (6), later shown to be in the outer coat (47), which appears to be surface exposed (20). CotB has been utilized as a vehicle for the presentation of heterologous antigens at the spore surface, suggesting its potential use in vaccine development (9, 20). Thus, the study of the assembly of CotB may allow a more precise manipulation of CotB as a fusion partner for heterologous antigen presentation. Also, it will expand our knowledge of the protein-protein interactions underlying assembly of a complex multiprotein structure and may provide us with tools for nanoengineering applications involving the *B. subtilis* spore. The *cotB* gene forms a cluster with two *cot* genes, *cotH* and *cotG* (6, 30, 36). Expression of *cotH* is under the control of  $\sigma^K$ , whereas both *cotG* and *cotB* are expressed later under the dual control of  $\sigma^K$  and GerE (18, 30, 36, 46, 45). Assembly of CotB-66 was shown to require expression of both *cotG* and *cotH* (30, 36).

We now show that *cotB* encodes a 46-kDa polypeptide (CotB-46) which is posttranslationally converted into a form of about 66 kDa (herein called CotB-66). This form of CotB (CotB-66) is equivalent to the 59-kDa protein previously reported by Donovan et al. (6). We show that formation of CotB-66 requires both *cotG* and *cotH* and that CotG does not accumulate in a *cotH* mutant. This suggests that the requirement for CotH or a CotH-dependent protein for CotB-66 formation results in part from its stabilization of CotG. We also found that CotB is present in complexes with CotG at the time when formation of CotB-66 is detected. Moreover, CotB was found to interact with itself and with CotG in a *Saccharomyces cerevisiae* two-hybrid assay. We suggest that formation of CotB-66 requires a direct interaction with CotG.

## MATERIALS AND METHODS

**Bacterial strains, media, and general techniques.** The *B. subtilis* strains used in this study are listed in Table 1. Sporulation was induced by nutrient exhaustion in Difco sporulation medium (31). *Pfu* polymerase (Stratagene) was used in all PCRs, and the cloned products were sequenced to ensure that no mutations were introduced. All other general methods were as described previously (5, 16, 17, 31, 38).

**Integrational vectors.** A fragment encompassing a chloramphenicol resistance (*Cm*<sup>r</sup>) gene was generated by PCR using primers *cat*-958D and *cat*-2040R (Table 2) and plasmid pHV33 as the template (41). The 1,080-bp PCR fragment was digested with *Eco*RI and *Pst*I and inserted between the same sites of pLITMUS 28 and pLITMUS 38 (New England Biolabs), creating the integrational vectors pMS38 and pMS39, respectively.

***cotB*, *cotG*, *ywrJ*, and *cotH* null mutants.** DNA from strain DL067 (obtained from the *Bacillus* Genetic Stock Center) (6) was used to construct AH141 (*cotB::cat*; Table 1). The *cotH* (AH1103) and *cotG* (AH1497) mutants (Table 1) have been described before (17, 48). To create an insertion within the 5' end of the *cotB* gene, a 395-bp PCR fragment (generated with primers *cotB*-7311D and

TABLE 1. Bacterial strains used in this study

Strain	Genotype/phenotype	Reference or source
MB24	<i>trpC2 metC3</i> /wild type/ <i>Spo</i> <sup>+</sup>	Laboratory stock
DL067 <sup>a</sup>	<i>cotB::cat</i> <sup>b</sup> / <i>Cm</i> <sup>r</sup>	6
AH140	<i>trpC2 metC3 cotA::cat</i> <sup>b</sup> / <i>Cm</i> <sup>r</sup>	29
AH141	<i>trpC2 metC3 cotB::cat</i> <sup>b</sup> / <i>Cm</i> <sup>r</sup>	This work
AH1103	<i>trpC2 metC3 cotH::cat</i> <sup>b</sup> / <i>Cm</i> <sup>r</sup>	48
AH1497	<i>trpC2 metC3 cotG</i> ΩpMS43 <sup>c</sup> / <i>Cm</i> <sup>r</sup>	17
AH1816	BL21 (DE3) (pLysS) (pMS8)	This work
AH1834	BL21 (DE3) (pLysS) (pMS16)	This work
AH2036	<i>trpC2 metC3 cotB</i> ΩpRZ24 <sup>c</sup> / <i>Nm</i> <sup>r</sup>	This work
AH2055	<i>trpC2 metC3 cotB</i> ΩpRZ29 <sup>c</sup> / <i>Nm</i> <sup>r</sup> , <i>Sp</i> <sup>r</sup>	This work
AH2088	<i>trpC2 metC3 ΔamyE::6xHis-cotB</i> / <i>Nm</i> <sup>r</sup>	This work
AH2089	<i>trpC2 metC3 ΔamyE::P<sub>cotE</sub> P<sub>1</sub>-cotB</i> / <i>Nm</i> <sup>r</sup>	This work
AH2119	<i>trpC2 metC3 ywrJ</i> <sup>b</sup> / <i>Cm</i> <sup>r</sup>	This work

<sup>a</sup> Strain DL067 was obtained from the *Bacillus* Genetic Stock Center as strain IS102.

<sup>b</sup> The *cotB*, *cotA*, *cotH*, and *ywrJ* alleles are the result of double-crossover events that replaced part of the chromosomal sequence with a chloramphenicol resistance determinant.

<sup>c</sup> The omega symbol (Ω) denotes that the plasmid was integrated via a single reciprocal crossover in the corresponding region of homology in the *B. subtilis* chromosome. Integration of pMS43 and pRZ29 resulted in the disruption of the *cotG* and *cotB* genes, respectively. Integration of pRZ24 creates an intact copy of the *cotB* gene under the control of its own promoter and fused at its 3' end to a six-His-encoding sequence.

*cotB*-7706R) (Table 2) was cloned into pCR 2.1-TOPO (Invitrogen) to create pRZ28. Next, the insert was released with *Eco*RI and *Sph*I and cloned between the same sites of pUS19 (3), yielding pRZ29. The single-reciprocal-crossover event (Campbell-type recombination) of pRZ29 at the *cotB* locus of strain MB24 (verified by PCR) produced the spectinomycin-resistant (*Sp*<sup>r</sup>) strain AH2055. To disrupt the *ywrJ* gene, a 780-bp PCR product obtained with primers *ywrJ*-125D and *ywrJ*-913R (Table 2) was digested with *Eco*RI and *Xho*I and inserted into pET30a(+) (Novagen) to yield pRZ47. A *Cm*<sup>r</sup> cassette was then released from pMS38 with *Hind*III and *Sna*BI and introduced between the *Hind*III and *Dra*I sites of pRZ47 to produce pRZ48 (Fig. 1). pRZ48 was cut with *Nde*I and *Xba*I and used to transform strain MB24 to *Cm*<sup>r</sup>, producing AH2119 (Table 1).

**Construction of strains carrying N- or C-terminal fusions of *cotB* to the six-His tag at the nonessential *amyE* locus.** A 1,424-bp PCR product encompassing the *cotB* promoter and coding region was generated with primers *cotB*-13D and *cotB*-1437R (Table 2) and cloned into pCR 2.1 TOPO to yield pRZ33. Plasmid pRZ33 served as a template to insert a six-His tag just downstream of the *cotB* initiation codon through the use of primers *cotB*-184Dhis and *cotB*-213RHis (QuickChange system; Stratagene) (Table 2). The modified *cotB* gene was released from the resulting plasmid (pRZ36) with *Eco*RI and *Hind*III and inserted between the same sites of the *amyE* integrational vector pMLK83 (21), thereby creating pRZ38. A neomycin-resistant (*Nm*<sup>r</sup>) transformant of strain MB24 with *Pst*I- and *Sca*I-linearized pRZ38 that was *AmyE*<sup>+</sup> (indicating a marker replacement event at *amyE*) was identified and named AH2088 (Table 1). To create a 3'-end fusion of *cotB* to the six-His tag, a *Cm*<sup>r</sup> cassette was released from pMS39 with *Spe*I and *Nhe*I and introduced into *Xba*I-digested pMS16 (see below). This produced pRZ24, whose Campbell-type integration into the *cotB* locus of strain MB24 generated AH2036 (Table 1).

**Construction of a strain expressing *cotB* from the *cotE* P1 promoter.** The *cotB* coding region and translation initiation signals were PCR amplified with primers *cotB*-150D and *cotB*-1373R (Table 2). The resulting 1,233-bp fragment was cleaved with *Eco*RI and *Xba*I and cloned downstream of the *cotE* P1 promoter in an *amyE* integrational vector (Costa and Henriques, unpublished results) derived from pMLK83 (21). This produced pRZ37, which (following digestion with *Sca*I) was used to transform strain MB24 with selection for *Nm*<sup>r</sup>. A *AmyE*<sup>+</sup> transformant was identified and named AH2089 (Table 1).

**Overproduction and partial purification of CotB.** Primers *cotB*-226D and *cotB*-1345R (Table 2) were used to PCR amplify the 1,120-bp *cotB* coding region, and the product was cleaved with *Nde*I and *Xho*I and inserted between the *Nde*I and *Sal*I sites of pET-30a(+) (Novagen) to yield pMS16. Primers *cotB*-235D and *cotB*-990R (Table 2) were used to PCR amplify a 756-bp fragment coding for the first 252 residues of CotB, which was cleaved with *Eco*RI and inserted between the *Eco*RI and *Eco*RV sites of pET-30a(+), creating pMS8. pMS16 or pMS8 was introduced into BL21(DE3) (pLysS) cells (Novagen), generating AH1834 or AH1816 (Table 1), respectively, in which the full-length CotB (CotB-FL) or its

TABLE 2. Primers used in this study

Primer	Sequence (5'-3')/restriction site or six-His tag <sup>a</sup>
<i>cat</i> -958D	GGGTAAGTACGCTGCAGGCAATAGTTACCC
<i>cat</i> -2040R	GGAGAAAGTCGAATTCAGAAAAAGAAGGATATGG
<i>cotB</i> -226D	GGAATTTTCATATGAGCAAGAGG/ <i>Nde</i> I
<i>cotB</i> -1345R	CCATTATCTACTCGAGTTTACGTTTCCAGTGATAG/ <i>Xho</i> I
<i>cotB</i> -235D	ATGAGCAAGAGGAGAATGAAATATC
<i>cotB</i> -990R	AATGAACGAATTCGCTGTCCTTATCAT/ <i>Eco</i> RI
<i>cotB</i> -13D	GCGAGTATATTTAAAAAGCTTTCACAATACC/ <i>Hind</i> III
<i>cotB</i> -1437R	CCTCAACATCTGTGTTAAGGAATTCATTCAAAC/ <i>Eco</i> RI
<i>cotB</i> -184Dhis	GGAGCAATTTGAATGCATCATCATCATCATAGCAAGAGGAGAATG/six-His tag
<i>cotB</i> -213Rhis	CATTCTCCTCTTGCTATGATGATGATGATGATGCATTCAAATTCCTCC/six-His tag
<i>cotB</i> -150D	GCAGTATGTTTATCATCTAGATATAAGTGACTAGG/ <i>Xba</i> I
<i>cotB</i> -1373R	CTGAAAAAGGGAATTCATTTATCTGATGCTCCTCC/ <i>Eco</i> RI
<i>cotB</i> -7311D	GGAGCAATTTGAATGAGCAAGAGGAG
<i>cotB</i> -7706R	GTTTAACGCAGCGTAATCATCTCCAGC
<i>ywrJ</i> -125D	GGAAACGTGAATCTAATAGATAATGGAGGAGC/ <i>Eco</i> RI
<i>ywrJ</i> -913R	GCCTTCTGCTTTTCTCGAGGGCAGTTTTGTATACACTAGGG/ <i>Xho</i> I
B/5/ <i>Nco</i>	GAGCCATGGGAATGAGCAAGAGGAGA/ <i>Nco</i> I
B/OMO/3	GCCTAGGATCCGGGCATCACTTTATC/ <i>Bam</i> HI
G/5/ <i>Nco</i>	GAGCCATGGAATTTGGGCCACTATTCC/ <i>Nco</i> I
G/ <i>Bam</i> /3	TACCTCCGCCGGGATCCTATTGAAAC/ <i>Bam</i> HI
H/5/ <i>Nde</i>	CTAAGGAGGACATATGATGAAGAATC/ <i>Nde</i> I
3/H/ <i>Bam</i>	CCGTGGATCCACCCATTTTCACGCAT/ <i>Bam</i> HI
H/5/ <i>Bam</i>	GTAAGGAGGGATCCGGATGAAGAAT/ <i>Bam</i> HI
H/3/ <i>Xho</i>	CGCGAATACCTCGAGCACGCATTCA/ <i>Xho</i> I

<sup>a</sup> Restriction sites or sequences encoding the six-His tag are underlined.

N-terminal half (CotBn) could be synthesized as C-terminal fusions to the six-His tag under the control of the *T7lac* promoter. Overproduction and partial purification of the His-tagged proteins was essentially as described previously (38). Both CotB-FL- and CotBn-6×His were used for the production of a rabbit polyclonal antiserum (Eurogentec, Herstal, Belgium). However, the antibody raised against CotB-FL was not very specific and the anti-CotBn antibody was used in all experiments herein reported.

**Generation of an anti-CotG antibody.** A peptide (CDDYKRHHDDYDSKKE) corresponding to residues 166 to 180 of the CotG primary structure (36) was synthesized and conjugated to keyhole limpet hemocyanin, and the conjugate was used to raise rabbit polyclonal antibodies against CotG (Eurogentec).

**Preparation of *B. subtilis* whole-cell extracts and immunoblotting.** *B. subtilis* whole-cell lysates were prepared, and immunoblotting experiments were conducted as described previously (39) except that gels for sodium dodecyl sulfate-12.5% or -15% polyacrylamide gel electrophoresis (SDS-12.5% or -15% PAGE) were used (as indicated in the figure legends). Antibodies were used at the following concentrations: anti-CotBn, 1:2,000; anti-CotG, 1:20,000; and anti-His (Novagen) (monoclonal), 1:50,000. Secondary anti-rabbit or anti-mouse antibodies (Sigma) were used at concentrations of 1:10,000 or 1:5,000, respectively.

**Spore purification and extraction of spore coat proteins.** Spores were harvested 24 h after the onset of sporulation and purified by density gradient centrifugation as described previously (16, 17). Proteins were extracted from purified spores and fractionated on SDS-12.5% or -15% PAGE gels. The gels were stained with Coomassie blue and then transferred to nitrocellulose for immunoblotting or to polyvinylidene difluoride membranes for N-terminal sequence analysis (16, 17).

**Ni<sup>2+</sup>-NTA affinity chromatography purification of complexes containing CotB-6×His.** Samples (100 ml) of Difco sporulation medium cultures of strain MB24 or AH2088 were collected at hour 8 of sporulation, and the cells were collected by centrifugation and resuspended in 1 ml of buffer A (1 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM 0.5% [wt/vol] NaCl, 2 mM phenylmethanesulfonyl fluoride). Lysates were prepared by passage (at 19,000 lb/in<sup>2</sup>) through a French press, and cell debris was removed by centrifugation (7,000 × *g* for 10 min at 4°C). The clarified lysate was applied to a Ni<sup>2+</sup>-NTA (nitrilotriacetic acid) agarose column (Qiagen) pre-equilibrated with 8 volumes of buffer A. The column was washed with 3 volumes of buffer A, and proteins were eluted with increasing concentrations of imidazole (10, 25, 50, 50, 100, 250, and 500 mM) in buffer A. Proteins in the different elution volumes were resolved on SDS-15% PAGE gels and subjected to immunoblot analysis with anti-CotBn or anti-CotG antibodies.

**Yeast two-hybrid analysis.** The coding regions of *cotB* and *cotG* were PCR amplified with primers B/5/*Nco* and B/OMO/3 and primers G/5/*Nco* and

G/*Bam*/3 (Table 2). The *cotB* and *cotG* PCR products were digested with *Nco*I and *Bam*HI and inserted between the same sites of both pAS2-1 and pACT2 (Clontech). The resulting plasmids are indicated in Table 3. The *cotH* coding sequence was obtained by PCR using two different sets of primers, primers H/5/*Nde* and 3/H/*Bam* and primers H/5/*Bam* and H/3/*Xho* (Table 2). The *cotH* PCR products were digested with *Nde*I and *Bam*HI and inserted between the same sites of pAS2-1 or with *Bam*HI and *Xho*I and fused to the GAL4 activation domain (AD) in pACT2 (see Table 3). Mating of *S. cerevisiae* strains and colony lift assays for detection of β-galactosidase activity were performed as described previously (33).

## RESULTS

***cotB* encodes a 46-kDa protein.** The *cotB* gene was previously identified (by reverse genetics) as encoding a spore coat

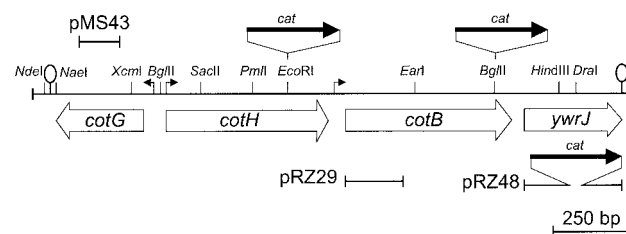


FIG. 1. Diagram of the *cotB* region of the chromosome. The horizontal line depicts a partial restriction map of the region (only relevant sites are shown for reference), and the arrows below the restriction map indicate the orientation and extent of open reading frames in the region. The positions of previously constructed insertional mutations in *cotG*, *cotH*, and *cotB* are shown above the restriction map. Disruption of *cotH*, *cotB*, or *ywrJ* resulted from double-crossover (marker replacement) events, whereas *cotG* was disrupted by the single-reciprocal (Campbell-type) integration of pMS43 (36). *cotB* was also inactivated by the Campbell-type integration of pRZ29. Right-angle arrows represent promoters. The *ywrJ* gene is presumably transcribed from the *cotB* promoter. Putative transcriptional terminators are represented by the stem-loop structures.



TABLE 3. Detection of *lacZ* transcription by colony lift assays of yeast strains expressing fusions of CotH, CotG, or CotB to the GAL4 activation and binding domains

AD fusion <sup>a</sup>	DNA-BD fusion <sup>b</sup>			
	pAS2-1 (BD)	pAS-B (CotB-BD)	pAS-G (CotG-BD)	pAS-H (CotH-BD)
pACT-2 (AD)	— <sup>c</sup>	—	—	—
pAC-B/(CotB-AD)	—	++	+	—
pAC-G/(CotG-AD)	—	+	+	—
pAC-H/(CotH-AD)	—	—	—	—

<sup>a</sup> Constructs based on pACT-2 which carry in-frame fusions of CotH, CotG, or CotB to the GAL4 AD. The plasmids were transformed into yeast strain Y190 (Clontech) before mating.

<sup>b</sup> Constructs based on pAS2-1 which carry fusions of CotH, CotG, or CotB to the GAL4 BD. The plasmids were transformed into yeast strain Y187 (Clontech) before mating.

<sup>c</sup> The time of development of blue color on a colony lift assay for the detection of *lacZ* expression is indicated as follows: ++, development of color in 1 h; +, development of color in up to 5 h. The symbol — indicates no color development in incubations of up to 24 h. Six independent colonies were tested for each combination.

component of about 59 kDa (6). Spores from a *cotB::cat* insertional mutant (strain DL067) (Fig. 1) exhibited the wild-type pattern of Coomassie-stained coat polypeptides on SDS-PAGE except for the absence of a 59-kDa polypeptide (6). Under our electrophoresis conditions, a band of about 66 kDa was absent from the coat of a mutant (AH141) bearing the same *cotB::cat* allele used by Donovan et al. (6) (Fig. 2, lane 2) or from spores of AH2055 (Fig. 2, lane 3, and Table 1), which bears an insertion of pRZ29 into the 5' end of *cotB* (Fig. 1). In addition, the N-terminal sequence of the corresponding band isolated from wild-type spores (Fig. 2, lane 1) was that deduced for the *cotB* product (data not shown). In two recent studies in which mass spectrometry techniques were used to identify spore- or coat-associated polypeptides, the *cotB*-encoded polypeptide was estimated to be 66 kDa (26) or 62 kDa (27). We infer that the 66-kDa protein (which we refer to as CotB-66) is equivalent to the protein found by Donovan et al. (6). Under our conditions, the closely migrating CotA protein (6) runs slightly slower than CotB, as shown by its absence from spores of a *cotA::cat* insertional mutant (AH140) (6, 29) (Fig. 2, lane 4).

Inspection of the *B. subtilis* genome sequence reveals, however, that *cotB* is capable of encoding a polypeptide of 380 residues and about 43 kDa (25). The disparity between the size of the *cotB*-dependent polypeptide associated with the coat and that calculated from the gene's sequence (43 kDa) could be due to abnormal electrophoretic mobility. Alternatively, CotB could be modified during its assembly into the spore coat. We reasoned that if migration of CotB in the 66-kDa region of the gel were due to its primary structure, CotB should still migrate at this position when produced in *E. coli*. Conversely, if the migration of CotB at 66 kDa were due to a modification related to the process of coat assembly, then this alteration of CotB could be restricted to *B. subtilis* cells. To investigate this, we first cloned the *cotB* gene with a six-His tag at its 3' end under the control of the T7lac promoter and introduced the resulting plasmid (pMS16) into cells of the *E. coli* host BL21(DE3) (Novagen) to produce strain AH1834 (Table 1). During construction of the CotB-6×His fusion, a

leucine codon and a glutamate codon were introduced at the 3' end of *cotB* (before the six-His-encoding sequence), representing 1.082 kDa. Thus, the CotB-6×His fusion protein has a predicted molecular mass of about 44 kDa. Induction of AH1834 with IPTG (isopropyl-β-D-thiogalactopyranoside) resulted in the production of polypeptides of about 46 kDa that accumulated as inclusion bodies. The 46-kDa polypeptide was solubilized in a urea-containing buffer and was partially purified by affinity chromatography on Ni<sup>2+</sup> columns (Fig. 2, lane 6). The N-terminal sequence (MSKRRMKY) confirmed that it derived from the *cotB* gene. When expressed in *E. coli*, therefore, *cotB* encodes a protein whose apparent molecular mass (46 kDa) is in good agreement with the size (43 kDa) predicted from the sequence of the gene (25). To directly compare the size of CotB-6×His made in *E. coli* to the size of the *cotB*-derived polypeptide that associates with the *B. subtilis* spore coat, we made use of strain AH2036 (Table 1), which resulted from the Campbell-type integration of pRZ24 and expressed a CotB-6×His fusion under the control of the *cotB* promoter. Addition of the six-His tag at the 3' end of CotB did not prevent its assembly into the coat structure (Fig. 2, lane 5) and did not significantly change the size of the protein associated with the coat relative to that of the wild-type protein (Fig. 2; compare lanes 1 and 5). We also note that an anti-six-His tag antibody reacted with a 66-kDa band in AH2036 spore coat extracts but not in extracts prepared from the wild-type strain MB24 (data not shown), suggesting that the C-terminal region of CotB is represented in the CotB-66 form.

The difference in size between the protein produced in

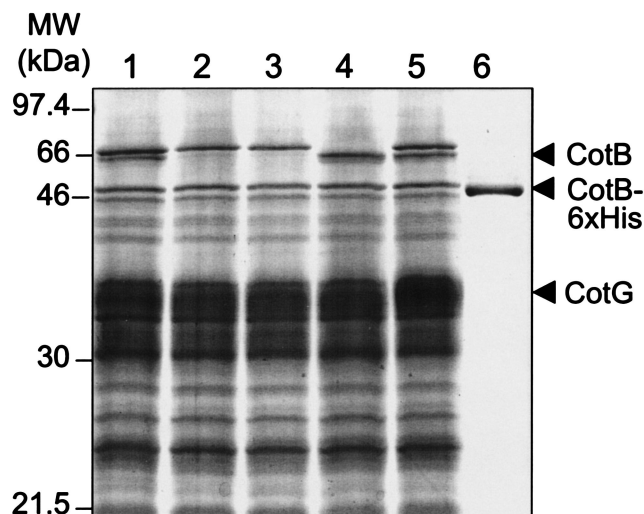


FIG. 2. *cotB* encodes a protein of 46 kDa in *E. coli* but accumulates as a 66-kDa form in the spore coat. Material extracted from purified spores of different *B. subtilis* strains (lanes 1 to 5; see Table 1) was resolved on an SDS-15% PAGE gel and stained with Coomassie blue. Lane 1, wild type (strain MB24); lane 2, *cotB::cat* (strain AH141); lane 3, *cotB::sp* (strain AH2055); lane 4, *cotA* (strain AH140); lane 5, CotB-6×His (strain AH2036). Lane 6 contains about 10 μg of CotB-6×His protein purified from an *E. coli* strain expressing a CotB-6×His fusion under the control of a T7lac promoter (strain AH1834; Table 1). The arrows mark the position of the CotB-46 or CotB-66 forms. The positions of molecular mass markers (MW) (in kilodaltons) are represented on the left side of the panel.

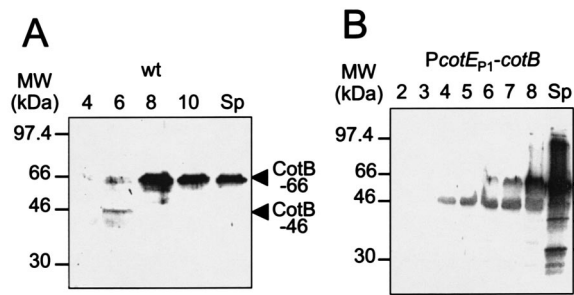


FIG. 3. Expression of *cotB* in sporulating cells. The synthesis of CotB was monitored in extracts from sporulating cells at the indicated times (in hours) after the onset of sporulation or in material extracted from purified spores (Sp) of the following strains (see Table 1): MB24 (wild type) (A) and AH2089 (which expresses *cotB* from the  $\sigma^E$ -dependent *cotE* P1 promoter) (B). CotB was detected by immunoblot analysis with antibodies raised against partially purified CotB. The arrows mark the positions of the CotB-46 and CotB-66 forms. The open arrowheads in panel B indicate the positions of putative CotB degradation products. The asterisk indicates the position of a form of CotB higher than 66 kDa. The positions of molecular mass markers (MW) (in kilodaltons) are shown on the left side of each panel.

*E. coli* and the polypeptide that accumulates in the *B. subtilis* spore coat prompts us to suggest that the form of CotB that prevails in the spore coat (CotB-66) bears a modification that increases its mass by about 20 kDa.

**CotB is synthesized as a 46-kDa species (CotB-46) during sporulation and is converted to a 66-kDa form (CotB-66).** To investigate whether CotB could also accumulate in *B. subtilis* as a polypeptide of about 43 to 46 kDa before being converted to the CotB-66 form, we used a polyclonal antiserum to follow its accumulation during sporulation. In whole-cell extracts prepared from strain MB24 cells harvested at hour 6 of sporulation (but not in those from the hour-4 sample), the antibody detected a species of approximately 46 kDa (Fig. 3A). This species has about the same size as the CotB-6 $\times$ His protein produced in *E. coli* (in good agreement with the predicted size of CotB). In the experiment documented in Fig. 3, this form of CotB (hereinafter designated CotB-46) was not detected in extracts prepared at hour 8 or 10 of sporulation or in spore coat extracts (Fig. 3A). In addition, the antibody detected a 66-kDa species in hour-6 extracts, presumably CotB-66 (see above) (Fig. 3A). In contrast to the results seen with CotB-46, the cellular level of CotB-66 increased from hour 6 to 8 and was the main form of CotB detected at hour 10 of sporulation or in purified coat material (Fig. 3A). The exact temporal profiles with which both CotB-46 and CotB-66 could be detected differed to some extent. In the experiment whose results are presented in Fig. 4A, for example, CotB-46 could still be detected at later times during sporulation whereas CotB-66 was only detected from hour 8 onwards. In any case, CotB-66 tended to predominate at later time points and was the major form of CotB detected in purified coat material. None of the CotB forms were detected in whole-cell extracts or in spore coat extracts prepared from sporulating cells or spores, respectively, of the AH141 (*cotB::cat*) or AH2055 (*cotB::sp*) mutants (data not shown).

The temporal pattern of accumulation of both the 46- and 66-kDa polypeptides is consistent with the time of expression of *cotB* during spore development which is induced around

hour 6 under the joint control of  $\sigma^K$  and GerE (18, 46). Recent results indicate that *cotB* expression may also be governed by the early mother cell-specific regulator  $\sigma^E$  (10). However, this early expression of CotB was not detected under our conditions. Our results suggest that CotB is synthesized around hour 6 of sporulation as a 46-kDa polypeptide (CotB-46) which rapidly undergoes a posttranslational modification to yield a 66-kDa form (CotB-66) which accumulates in the spore coat.

**Synthesis and modification of CotB can be uncoupled in sporulating cells.** To independently test whether CotB could be synthesized and accumulate as a 46-kDa form in *B. subtilis*, we placed the *cotB* coding region under the control of the strong *cotE* P1 promoter ( $P_{cotE P1}$ ), which is utilized by the  $\sigma^E$ -containing form of RNA polymerase (46). We reasoned that the putative modification of CotB could depend on other late-expressed genes and that the expression of *cotB* at sufficient levels prior to the onset of  $\sigma^K$  activity could confirm the size of unmodified CotB in *B. subtilis*. The  $P_{cotE P1}$ -*cotB* fusion was inserted at the *amyE* locus in a *cotB* null mutant, and the accumulation of CotB was monitored in AH2089 by immunoblot analysis throughout sporulation and in purified coat material. Control experiments have shown that the insertion of *cotB* at the *amyE* locus resulted in the production and assembly of CotB in a manner indistinguishable from that seen with the wild-type strain MB24 (data not shown). When *cotB* was expressed from the *cotE* P1 promoter, CotB was detected as a 46-kDa species from hour 4 of sporulation onwards (Fig. 3B).

Thus, as seen with *E. coli* cells, *cotB* seems to encode a protein of about 46 kDa. Even though *cotB* was expressed at least 2 h earlier than normally, interestingly, CotB-66 was detected only from hour 6 of sporulation onwards, as seen with the wild-type strain MB24 (Fig. 3A). We also found in the coats of AH2089 at least two relatively abundant bands of about 40 and 34 kDa, which we interpret as stable degradation forms of CotB (Fig. 3B and 4B). Donovan et al. (6) also described a coat-associated polypeptide of 34 kDa whose N-terminal sequence matched that of CotB. Inspection of the

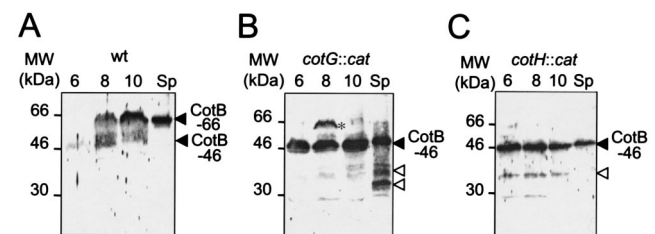


FIG. 4. Expression of both *cotG* and *cotH* is required for the efficient formation of CotB-66. The figure illustrates the accumulation of CotB at the indicated times (in hours) after the onset of sporulation and in spore coat extracts (Sp) in the following strains (Table 1): MB24 (wild type) (A), AH1497 (*cotG::cat*) (B), and AH1103 (*cotH::cat*) (C). The accumulation of CotB was monitored with a polyclonal antibody raised against the partially purified protein produced in *E. coli* cells. The arrowheads on the right sides of the panels indicate the positions of CotB-46 and CotB-66. The open arrowheads in panels B and C indicate the position of putative CotB degradation products. The asterisk in panel B indicates the position of a band of about 66 kDa that accumulates transiently around hour 8 of sporulation in the *cotG* mutant. The positions of protein molecular mass markers (MW) (in kilodaltons) are indicated to the left of each panel.

*B. subtilis* genome sequence indicates that this polypeptide cannot be the product of a separate gene (25). Since the original insertion into *cotB* (close to its 3' end) (Fig. 1) did not prevent its accumulation, the 34-kDa form must derive from the N-terminal portion of the CotB protein as initially hypothesized (6). Presumably this form of CotB represents a stable degradation product of higher-molecular-mass forms of CotB. Spores of AH2089 ( $P_{cotE}$   $P_1$ -*cotB*) show wild-type resistance to heat and lysozyme, and except for the differences mentioned above (which were detected by immunoblot analysis), the collection of extractable coat polypeptides does not differ from that of wild-type spores as judged by Coomassie staining (data not shown). However, while spores of the *cotB::sp* mutant AH2055 are not impaired in their ability to germinate in response to L-alanine (as also reported for a strain bearing the *cotB::cat* allele) (6), AH2089 spores are slow to germinate upon exposure to L-alanine (data not shown). This suggests that the timing of the expression of *cotB* is important for the spore's response to the germinant L-alanine.

Transcription of *cotB* normally requires both  $\sigma^K$  and GerE (18, 46). That expression of *cotB* is not sufficient for formation of CotB-66 suggested that at least one additional late gene is needed for the formation of CotB-66. We cannot exclude that formation of CotB-66 involves a factor synthesized early in the mother cell line of gene expression (under the control of  $\sigma^E$ ), but in any case CotB-66 is only formed late, coincidentally with the onset of  $\sigma^K$ -directed gene transcription. Thus, irrespective of the involvement of early gene products, formation of CotB-66 appears to be directly or indirectly dependent on the products of late,  $\sigma^K$ -dependent genes or morphogenetic events. We also note that in the  $P_{cotE}$   $P_1$ -*cotB* strain, CotB-46 accumulates at later times during sporulation and undergoes assembly into the spore coat (Fig. 3B). It appears that in the  $P_{cotE}$   $P_1$ -*cotB* strain, CotB-46 cannot be completely converted into the 66-kDa form. Alternatively, the protein at 46 kDa that accumulates from hour 6 onwards may result from degradation of CotB-66. In any event, we infer that formation of CotB-66 is not a strict requirement for assembly of CotB (see also below).

**Formation of CotB-66 requires expression of both *cotG* and *cotH*.** Previous work has shown that assembly of CotB (CotB-66) into the spore coat requires expression of both the *cotG* and *cotH* loci (30, 36). The *cotG* gene is found just upstream and divergently oriented with respect to *cotH*, whereas *cotB* immediately follows *cotH* (25, 30, 36) (Fig. 1). Both the *cotG* and *cotH* genes are under the control of  $\sigma^K$ , but the expression of *cotG* further depends on *gerE* (30, 36). To determine whether *cotH* or *cotG* was required for the modification of CotB, we used our polyclonal antibody to monitor the synthesis of CotB in *cotH* and *cotG* null mutants (AH1103 and AH1497, respectively) (Table 1). In contrast to the results seen with the wild type (Fig. 4A), we found CotB-46 to be the predominant form of CotB in whole-cell extracts of a *cotG* mutant prepared 6, 8, or 10 h after the onset of sporulation (Fig. 4B). We found CotB-66 (or a species of approximately the same size) to accumulate transiently at hour 8 in the *cotG* mutant AH1497, but even in this sample CotB-46 was more abundant (Fig. 4B). At present we do not know whether this species is the same as the CotB-66 form seen in the wild type (see also Discussion). Moreover, CotB-46 was the predominant form of CotB found

in coat extracts prepared from *cotG* spores (Fig. 4B). Several degradation products of CotB were found to accumulate in cells or spores of the *cotG* mutant (Fig. 4B). We then investigated the accumulation of CotB in sporulating cells and in the coats of the *cotH* mutant AH1103 (Table 1). The results presented in Fig. 4C show that (together with what appear to be degradation products) only CotB-46 is detected in whole-cell extracts of sporulating cells or in material purified from the coats of *cotH* mutant spores. Therefore, expression of *cotH* is also a requirement for the formation of CotB-66. However, CotB-66 was never detected in the *cotH* mutant, suggesting that *cotH* and *cotG* might have different roles in the formation of CotB-66. In any case, the results implicate both *cotH* and *cotG* in the formation of CotB-66. We cannot ascertain whether *cotH* and *cotG* are also involved in the assembly of CotB-66, but we note that the expression of neither *loci* is required for the association of CotB-46 with the coat structure.

The *cotB* gene is followed by *ywrJ* (25) (Fig. 1), and the two genes appear to be cotranscribed during sporulation (10). To test whether *ywrJ* had any role in spore coat assembly, we created a *ywrJ* deletion mutant (AH2119) (Table 1) by a marker replacement event involving plasmid pRZ48 (Fig. 1). We found that disruption of *ywrJ* did not cause any detectable effect on the coat polypeptide composition, heat or lysozyme resistance of the resulting spores, or accumulation of CotG or CotB-66 (data not shown). Thus, the function of the *ywrJ* gene remains unknown.

**CotH is required for the accumulation of CotG.** The need for both *cotH* and *cotG* for the formation of CotB-66 could indicate that the two *loci* act together or could reflect a hierarchical control of one locus over the other. To begin to investigate these questions, we first tried to overproduce CotG in *E. coli* for antibody production. In spite of many attempts to overproduce CotG as N- or C-terminal fusions to the six-His tag or to partners such as maltose binding protein or glutathione S-transferase, we never observed accumulation of the fusion proteins in *E. coli* (data not shown). We therefore decided to raise a rabbit polyclonal antibody against a peptide derived from residues 166 to 180 of the CotG primary structure (see reference 36 and Materials and Methods). The *cotG* gene codes for a protein with 195 amino acid residues with a predicted molecular mass of 24 kDa (36). However, CotG is seen mainly as a wide, diffuse band of about 36 kDa in spore coat gels (Fig. 1; see also below) (17, 30, 36). The discrepancy between the predicted size and the observed size of CotG has been attributed to its unusual primary structure, which bears a central region of 117 amino acids organized in nine repeats of a 13-amino-acid sequence motif (36). Using the anti-peptide antibody, we detected CotG as diffuse bands of about 32 and 36 kDa in whole-cell extracts from hour 8 of sporulation onwards (Fig. 5, lane 8 [wild-type strain results]). Migration of the 36-kDa species appears to coincide with that of the 36-kDa *cotG*-dependent band seen in Coomassie-stained coat gels (Fig. 5, lane SpC [wild-type strain results]) (17, 30, 36). The 32-kDa band, which is the band that is the closest in size to the expected size of the *cotG*-encoded protein (24 kDa), also coincides with a band seen in the Coomassie-stained gel of the same coat protein extract (Fig. 5, lanes Sp and SpC [wild-type strain results]). Additional higher-molecular-mass forms of CotG (of about 70 and over 100 kDa) were found in hour-10



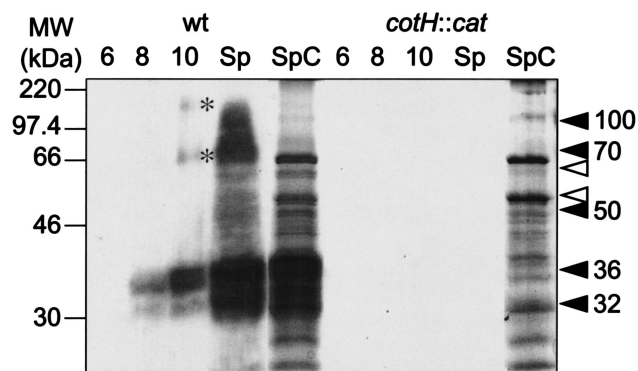


FIG. 5. Accumulation of CotG in sporulating *B. subtilis*. The figure depicts the immunoblot analysis of the accumulation of CotG at the indicated times (in hours) after the onset of sporulation in spore coat extracts (Sp) in the wild-type strain (strain MB24) (Table 1) or in a *cotH::cat* mutant (AH1103). CotG was detected with a polyclonal antibody raised against a peptide derived from residues 166 to 180 of the CotG primary sequence (see Materials and Methods). For comparison, the lanes marked SpC (for strain MB24 or AH1103) show a Coomassie-stained gel of the same sample of extracted coat proteins used for the immunoblot analysis (lanes marked with Sp). Black arrowheads on the right side of the panel indicate the positions of forms of CotG of about 100, 70, 50, 36, and 32 kDa. Other, less-abundant forms of CotG are indicated by open arrowheads. Asterisks indicate a form of CotG of over 100 kDa that was predominantly found in whole-cell extracts at hour 10 of sporulation and a form of about 70 kDa that was found both in the hour 10 sample and in purified coat material (Sp). The positions of protein molecular mass markers (MW) (in kilodaltons) are indicated on the left side of the panel.

whole-cell extracts (Fig. 5). In purified coat material, five main forms (around 32, 36, 50, and 70 and over 100 kDa) of CotG were detected (spore coat extract from wild-type spores) (Fig. 5). Presumably, some of these species are the same as those detected in hour-10 extracts. The antibody also revealed several less-distinct CotG forms between the 100- and 32-kDa species found in spore coat extracts (Fig. 5), which could have been caused by extensive cross-linking of CotG or proteolysis or a combination of the two processes. None of the bands seen in immunoblotting analysis of wild-type cell lysates or coat material were detected in extracts of a *cotG* insertional mutant, demonstrating the specificity of the antibody (data not shown). We then examined the accumulation of CotG in a *cotH* mutant. Unexpectedly, we did not detect any forms of CotG in whole-cell lysates prepared from sporulating cultures or in material extracted from purified spores of the *cotH* mutant AH1103 (Fig. 5, lanes 6 to 10 and Sp [*cotH::cat* mutant results]). Analysis (by SDS-PAGE and Coomassie staining) of the profiles of proteins extracted from AH1103 spores (Fig. 5, lane SpC [*cotH::cat* mutant results]) revealed the pattern expected for the *cotH* mutant (30). Since the *cotH::cat* mutation does not affect transcription of the *cotG* gene (30), these observations strongly suggest that CotG is unstable and that CotH or a protein controlled by CotH is required for the accumulation of CotG. In any case, the need for *cotH* expression for the formation of CotB-66 may derive in part from its requirement for the accumulation of CotG. The possibility that CotH has, in addition, a more direct role in formation of CotB-66 is not excluded.

**CotB and CotG form complexes in vivo.** The observation that *cotH* and *cotG* promote formation of CotB-66 prompted us to investigate possible associations in vivo. We used a *B. subtilis* strain expressing a CotB-6 $\times$ His fusion protein at the *amyE* locus (strain AH2088) to purify (by affinity chromatography) complexes containing CotB. We prepared whole-cell extracts from AH2088 cells at hour 8 of sporulation and applied the extract to a Ni<sup>2+</sup>-NTA agarose column (Fig. 6). The column was first washed and was then eluted with increasing concentrations (from 10 to 500 mM) of imidazole (see Materials and Methods). The proteins were electrophoretically resolved, and the presence of CotB or CotG in the various fractions was assessed by immunoblot analysis. We do not presently have a good antibody against CotH and could not test whether this protein also copurified with CotB. We found that most of the CotB present at hour 8 of sporulation did not bind to the Ni<sup>2+</sup>-agarose column and that it was detected in the column flowthrough (Fig. 6A, lane 1). Part of this CotB is

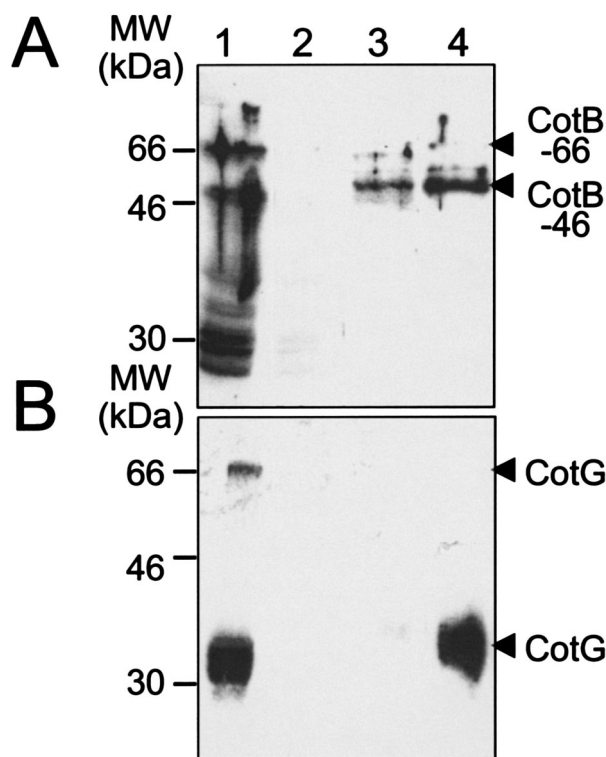


FIG. 6. CotB and CotG are present in complexes in vivo. Whole-cell extracts were prepared from the wild-type (MB24) or AH2088 (CotB-6 $\times$ His) strain at hour 8 of sporulation. The extracts were loaded on a Ni<sup>2+</sup>-NTA-agarose column, and the presence of CotB (A) or CotG (B) in the column flow through or eluate fractions was monitored by immunoblot analysis with specific antibodies. After loading, the column flow through was collected and the column was washed and eluted with increasing concentrations of imidazole (see Materials and Methods). The figure represents the results of analysis of the following samples: the column flowthrough (lane 1) and the 50 mM (lane 3) and 100 mM (lane 4) imidazole eluate fractions after application of the AH2088 extract and the 100 mM imidazole fraction resulting from a similar experiment using an extract prepared from the wild-type strain MB24 (untagged *cotB*) (lane 2) (Table 1). Molecular mass markers (in kilodaltons) are indicated on the left side of the panels, and the positions of CotB-46, CotB-66, and CotG are indicated on the right side.

likely to result from expression of the wild-type *cotB* gene in AH2088 (Table 1), suggesting that untagged CotB is not retained by the column. To verify this, we prepared a whole-cell lysate from a sporulating culture of strain MB24 at hour 8 of sporulation. The lysate was applied to a  $\text{Ni}^{2+}$ -NTA column, and the column was eluted with a step gradient of imidazole at concentrations up to 500 mM. No CotB was found in the elution fractions by immunoblot analysis. We conclude that under these conditions, untagged CotB is not retained by a  $\text{Ni}^{2+}$  column (data not shown). In strain AH2088 (CotB-6 $\times$ His), some CotB did bind to the column and was eluted at imidazole concentrations of 50 mM (lane 3) and 100 mM (lane 4). Most of the fusion protein retained by the affinity column was the 46-kDa form (Fig. 6A, lanes 3 and 4). Very little CotB-66 was found in the 50 or 100 mM imidazole fractions, but this form of the protein appeared to be slightly more abundant in the 50 mM fraction (Fig. 6A, lanes 3 and 4). No CotB was detected upon elution of the column with buffers containing higher imidazole concentrations (up to 500 mM; data not shown).

We then subjected samples of the same fractions to immunoblot analysis using an anti-CotG antibody (Fig. 6B). We found that a fraction of CotG present in whole-cell extracts prepared at hour 8 of sporulation (and that included at least two forms [about 36 and 70 kDa] of the protein) was not retained by the column (Fig. 6B, lane 1). However, CotG (mainly a form of about 36 kDa) also coeluted with CotB-46 at an imidazole concentration of 100 mM (Fig. 6B, lane 4). No other column fractions (at imidazole concentrations up to 500 mM) contained CotG. Control experiments using the wild-type strain MB24 (Table 1) demonstrated that CotG was not retained by the column when CotB did not carry a six-His tag (Fig. 6B, lane 2, and data not shown). We do not presently know why CotB elutes at two different imidazole concentrations or why CotG only coelutes with CotB at the higher (100 mM) imidazole concentration. Presumably, CotB exists in several forms that interact differently with the column. In any case these results indicate that CotB copurifies with CotG, and we infer that CotB (perhaps mostly as CotB-46) and CotG are present in complexes in vivo.

**CotG and CotB directly interact.** Since CotG and CotB appeared to form complexes in sporulating cells, we wanted to investigate whether the two proteins could interact directly. In addition, we wanted to test whether CotH could interact with either CotG or CotB. Interactions among CotH, CotG, and CotB were tested in vivo using a yeast two-hybrid system (12, 13, 14). We fused the entire coding sequence of *cotB*, *cotH*, or *cotG* to either the AD in pACT-2 or the DNA-binding domain (DNA-BD) in pAS2-1 of the yeast transcriptional activator GAL4, and combinations of the fusion plasmids and/or vectors were introduced into appropriate yeast reporter strains (33) (Table 3). Interaction of the fusion proteins in the yeast strains resulted in the expression of a *lacZ* reporter gene and was detected by a colony lift assay (see Materials and Methods). As shown in Table 3, no  $\beta$ -galactosidase activity was detected when individual fusions were expressed with the corresponding control vector. In addition, background levels (in yeast cells carrying both vectors) were found to be negligible (Table 3). Using this system, we detected interactions between CotB and itself and between CotG and itself. Additionally, we detected

an interaction between CotB and CotG (Table 3). All of the interactions were found when the involved proteins were fused to either the GAL4 AD or the GAL4 BD (Table 3). In contrast, we did not find evidence for an interaction between CotH and either CotG, CotB, or itself. These results suggest that CotB is capable of associating with itself and also with CotG and that CotG is also capable of self-association.

## DISCUSSION

We found that *cotB* encodes a polypeptide of about 46 kDa (CotB-46) that (soon after synthesis around hour 6 of sporulation) undergoes a posttranslational modification that converts it into a 66-kDa form (CotB-66). The CotB-66 polypeptide is the form of CotB previously found in the spore coat by Donovan et al. (6). Under our conditions, CotB-66 is the main form of CotB present in the spore coat. That *cotB* codes for a 46-kDa protein is supported by two lines of evidence. First, expression of *cotB* in *E. coli* results in the accumulation of a 46-kDa polypeptide. Second, under genetic conditions in which synthesis of CotB in *B. subtilis* was artificially induced from the early  $\sigma^E$ -dependent *cotE* P1 promoter (46) a 46-kDa polypeptide accumulated from hour 4 of sporulation onwards. Conversion of CotB-46 into CotB-66 in the *P<sub>cotE</sub> P1-cotB* strain only occurred around hour 6 of sporulation, as seen with the wild type, indicating the involvement of other, late-expressed genes in the modification of CotB-46. Indeed, our results indicate that the expression of both *cotH* (which is driven by  $\sigma^K$ ) (30) and *cotG* (which requires  $\sigma^K$  and GerE) (36) is needed for the formation of CotB-66. The data also indicate that the involvement of *cotH* and *cotG* in CotB-66 formation results in part from a hierarchical sequence of events. In fact, CotG does not accumulate in the absence of CotH, suggesting that the latter is an unstable protein and that CotH or a CotH-dependent factor is somehow needed to stabilize CotG. Even though we did not find evidence for a direct interaction between the two proteins in a yeast two-hybrid system, CotH might act as a chaperone (binding directly to CotG) to promote its stabilization. We note that CotH is found only in minute amounts in the final coat structure (48), whereas CotG accounts for a significant proportion of the protein extractable from the coat of wild-type spores (7, 15). Chaperones play essential roles in the macromolecular assembly of other biological structures (see, for example, references 1 and 37) but have never been proposed to play such roles in the context of the assembly of the bacterial endospore coat. An alternative explanation for the role of CotH is that CotH or a CotH-dependent protein acts to inhibit a protease that uses CotG as a substrate.

Previous work has shown that in addition to CotH, CotG and CotB are absent from *cotH* spores (30). In contrast, in addition to CotG, only CotB (CotB-66) is missing from *cotG* spores (36). The *cotH*-dependent stabilization of CotG helps to explain its requirement for the assembly of CotG, whereas the *cotG*-dependent assembly of CotB is in part explained by the role of CotG in CotB-66 formation (Fig. 7). We do not know whether CotG is directly recruited by CotH to the coat structure or whether the assembly of CotB-66 is directly controlled by CotH or CotG (Fig. 7). However, the observation that CotB-46 accumulates in the coats of *cotH* or *cotG* mutants indicates that the assembly of at least this form of the protein



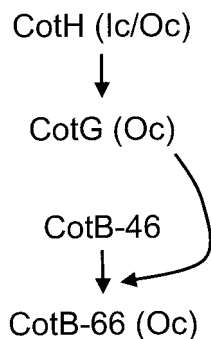


FIG. 7. Assembly of CotB. Previous work has shown that CotH is needed for the assembly of CotG and CotB (30, 36). The results of the present study indicate that *cotB* encodes a protein of about 46 kDa which is rapidly converted into a form of about 66 kDa. The results also indicate that CotH is required directly or indirectly for the stabilization of CotG, which in turn promotes the conversion of CotB-46 into the CotB-66 form, possibly via a direct interaction with CotB-46. It is not known whether CotH directly controls the assembly of CotG or whether CotG is directly involved in the recruitment of CotB-66. However, assembly of CotB-46 can occur in the absence of CotH or CotG. Where known, the location of the indicated proteins within the coat structure is indicated (Oc, outer coat; Ic/Oc, inner coat-outer coat interface).

is independent of the presence of CotH and CotG. Lastly, we note that CotB-66 is detected transiently in the *cotG* mutant but not in *cotH* cells, suggesting that other than its role in the stabilization of CotG, CotH might serve yet another role in the formation of CotB-66. However, it is also possible that this band is a cross-reactive species that only accumulates in a *cotG* mutant.

CotG is first detected around hour 8 of sporulation as two main antigenic forms of about 32 and 36 kDa (Fig. 5). The 32-kDa form could represent the unmodified product of the *cotG* gene (24 kDa) whose abnormal migration may be attributed to its unusual primary structure (36). CotG appears to be converted into forms of about 36 and 70 kDa in sporulating cells and then to be subjected to extensive cross-linking as it is assembled into the spore coat. That CotG is able to form cross-linked multimers has been previously suggested on the basis of the results of analysis of the coat structure in *sodA* mutant cells (17) and is supported by the results of the yeast two-hybrid assay (this work). A more detailed characterization of the synthesis and assembly of CotG is currently under way.

Our finding that CotG copurifies with CotB strongly suggests that the two proteins are present in complexes at the time in coat assembly during which formation of CotB-66 is detected. In that respect it is noteworthy that CotG (a 36-kDa form of the protein) appears to copurify mainly with CotB-46. On the basis of the results of the yeast two-hybrid experiments, it is tempting to suggest that CotG may directly interact with CotB-46 during assembly of the spore coat to promote formation of CotB-66 (Fig. 7). It is unknown whether CotH also copurifies or interacts with CotB, just as it is unknown whether the presence of CotG and CotH is sufficient to promote the modification of CotB. Spores produced by *cotB* and *cotG* mutants are not affected in their resistance to lysozyme or germination properties (6, 36). Therefore, the significance of the interaction between CotB and CotG is presently unclear and

may only be apparent under conditions that have not yet been identified.

The nature of the posttranslational modification of CotB remains unclear. On the basis of the size difference from the unmodified form and of the observation that the CotB-66 form presents only one N-terminal sequence (6; this work), the easiest explanation is that CotB-66 is a cross-linked homodimer with irregular mobility (the expected size would be of 92 kDa [in contrast to the observed size of 66 kDa]). It is also possible that CotB-66 is a homodimer cleaved near the C terminus of the protein, but, if so, our observation that a C-terminal six-His sequence is present in the 66-kDa form indicates that at least one of its components is the full-length protein. Our finding that CotB is capable of self-interaction in a yeast two-hybrid system supports the suggestion that CotB undergoes multimerization. Alternatively, CotB-66 is a heterodimer, perhaps containing CotG (given that the two proteins appear to interact); in that case, however, either the cross-linking involves the N terminus of the second component or its N-terminal sequence is blocked and refractory to analysis. Several types of covalent cross-links have been proposed or detected in the coat. These include disulfide bridges (note, however, that *cotB* does not code for any cysteine),  $\gamma$ -glutamyl-lysyl isopeptide bonds mediated by a coat-associated transglutaminase (see, for example, references 16, 22, 23, and 44), or *o,o*-dityrosine cross-links (reference 17 and references therein). Work in progress aims at determining the nature of the posttranslational modification of CotB and its structural significance as well as the protein components involved and their roles.

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